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TECHNICAL REPORT
FD-22

A STUDY OF CHEMICAL CHANGES PRODUCED BY HEAT AND BY IRRADIATION OF MEAT AND MEAT FRACTIONS

by
W. A. LANDMANN

SEPTEMBER 1965

AMERICAN MEAT INSTITUTE FOUNDATION
Chicago, Illinois 60637

Contract No. DA19-129-QM-1972

U. S. ARMY NATICK LABORATORIES
Natick, Massachusetts

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U. S. Army Materiel Command
U. S. ARMY NATICK LABORATORIES
Natick, Massachusetts 01762

FOREWORD

The acceptance of meat preserved by ionizing radiation is less than that required for military use because of a characteristic, undesirable odor developed during processing. Identification of the precursor of this odor and the mechanisms of its formation would provide knowledge useful in developing means to overcome it, thereby increasing the acceptance of radiation sterilized meat items.

The work covered in this report, performed by the American Meat Institute Foundation under Contract No. DA 19-129-QM-1972 represents an attempt to isolate and identify one precursor of irradiation odor. The investigator was W.A. Landmann. His collaborators were Othmer F. Batzer, Arlene T. Santoro, Edith M. Olson and Robert I. Morrow.

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TABLE OF CONTENTS

ABSTRACT

The experiments described herein are attempts at identification of the precursor material which gives rise to a specific irradiation odor in beef muscle tissue when subjected to a sterilizing radiation dose (5 megarad).

Early work indicated that this irradiation odor was associated with the phospholipid fraction of irradiated meat. Experiments using Thin Layer Chromatography, infra-red analysis, and chemical tests, indicate that sphingomyelin or some similar compound could be the precursor material.

Efforts to identify the odor by use of infra-red analysis, gas chromatography, and chemical tests were unsuccessful.

Efforts to reproduce the odor by peroxide oxidation or ozonolysis of sphingomyelin were successful except that other odors were produced in much greater intensities.

Irradiation of sphingomyelin and sphingosine sulfate did not produce the irradiation odor but this could possibly be attributed to differences in conditions.

The possibility of a sphingomyelin containing a specific fatty acid linked through the amid nitrogen should not be overlooked as the source of the irradiation odor.

A STUDY OF CHEMICAL CHANGES PRODUCED BY HEAT AND BY IRRADIATION OF MEAT AND MEAT FRACTIONS

The work covered by this report describes attempts to isolate and identify the precursor material which forms a specific irradiation odor when beef muscle tissue is subjected to a sterilizing radiation dosage. Previous work (1) indicated that this irradiation odor was contained in the phospholipid material in irradiated beef muscle tissue. During the course of this work the isolation procedure as described in (1) was modified. These modifications, and the methods used in attempts to identify both the precursor material, and the nature of the odor will be described under the appropriate headings.

Isolation Procedures:

In the original isolation procedure (1), irradiated (5 megarad) ground beef was washed with aliquots of distilled water until the residue was essentially colorless. Usually five to six washings with filtration through cheese cloth, were necessary. The residue was then freeze-dried, and extracted overnight in Soxhlet extractors with hexane. The residue was then air-dried, and re-extracted overnight with 2:1 chloroform-methanol in Soxhlet extractors. Although there was no detectable removal of the irradiation odor from the residue by hexane, the chloroform-methanol extraction removed it completely.

On removal of the chloroform-methanol under vacuum (water jet aspirator), a crude phospholipid fraction was obtained, which contained the irradiation odor. (Addition of water is necessary to produce the odor.) Total yield of the crude fraction was around 25 g. from 2000 g. muscle tissue. The crude material was separated on a silicic acid column using chloroform as the stationary phase and eluting with a gradient concentration of methanol. A diagram of the set up for this procedure is shown in (1). The silicic acid column separation was a limiting factor in obtaining sufficient material for analysis. Despite the relatively large size of the column, only 2 g. of the crude material could be applied at any one time. The run usually required 2 to 3 days, and the yield of the fraction containing the irradiation odor was usually 10 to 25 mg. This fraction contained at least seven components and contained a much greater concentration of the irradiation odor than the crude material.

Over a period of time various short cuts were devised in the extraction procedure. First, it was found that if the freeze-dried residue was placed in a large flask and soaked in the various solvents used, it was unnecessary to use Soxhlet extractors. The new procedure was as follows: The freeze-dried residue was placed in a large flask, covered with hexane and allowed to stand overnight. The next morning the hexane was filtered off and the residue briefly washed with another aliquot of hexane. The residue was air-dried and extracted in the same manner with chloroform. After the residue was again dried, the same procedure was followed using methanol. By this procedure, the neutral

lipids were removed by hexane as effectively with Soxhlet extraction. The Chloroform extract removed a large amount of material, mostly of a lecithin nature, without removing the irradiation odor. The methanol completely removed the irradiation odor from the residue, in a form which appeared to contain considerably smaller amounts of other contaminating substances than did the material from the previous procedure.

The silicic acid column was also a limiting factor in obtaining material for tests. In investigations designed to increase the yield, it was found that by mixing the methanol extract with an equal volume of water, and adding hydrochloric acid to bring the resulting solution to approximately 0.5 normal, a precipitate appeared after three days at room temperature. This precipitate contained a strong irradiation odor. This material was used throughout later experiments in attempts to identify the precursor material and the substance(s) having the irradiation odor: The procedure used is given in Figure 1.

Methods used in attempts to identify precursor substance and odor.

Thin Layer Chromatography:

In earlier work, the methanol extract was spotted on silica gel-G plates and run in butanol-acetic acid-water (12:3:5 v/v). The irradiation odor was located by scraping the silica gel away from one edge of the plate, wetting and smelling the exposed area. Various reagents were then used to locate specific phospholipid material on the chromatograms: Dragendorf's solution (BiI_4) for compounds containing choline, ninhydrin solution for serine compounds, diphenylamine for carbohydrates, ammonium molybdate for phosphates, and Schiff's reagent for aldehydes. Results are depicted in Figure 2. The area in which the irradiation odor was located gave positive tests with Dragendorf's solution, ammonium molybdate and Schiff's reagent. Silica gel in the area containing the irradiation odor was removed from the untreated portions of the plates and eluted with a small amount of methanol. The methanol eluent was spotted on a fresh silica gel plate, run in chloroform-methanol-water (65:25:4 v/v) and treated as before. Results are also shown in Figure 2. On this chromatogram the irradiation odor was located in an area that was negative to the applied reagents. While phosphate was not tested directly on this chromatogram, the methanol eluent was checked, prior to its application on the plate, by a more sensitive method (2). The test was negative. The above results were duplicated many times.

Since the acid precipitation procedure led to a more concentrated irradiation odor, this precipitate was dissolved in methanol and used on TLC plates in the systems described in the preceding paragraphs. With both solvent systems the irradiation odor remained at the base line along with material that gave a positive Dragendorf's test, positive phosphate, and positive Schiff's test.

Efforts were made to find solvent systems to move this material

Figure 1.
Modified Procedure for Isolation
of Irradiation Odor Material

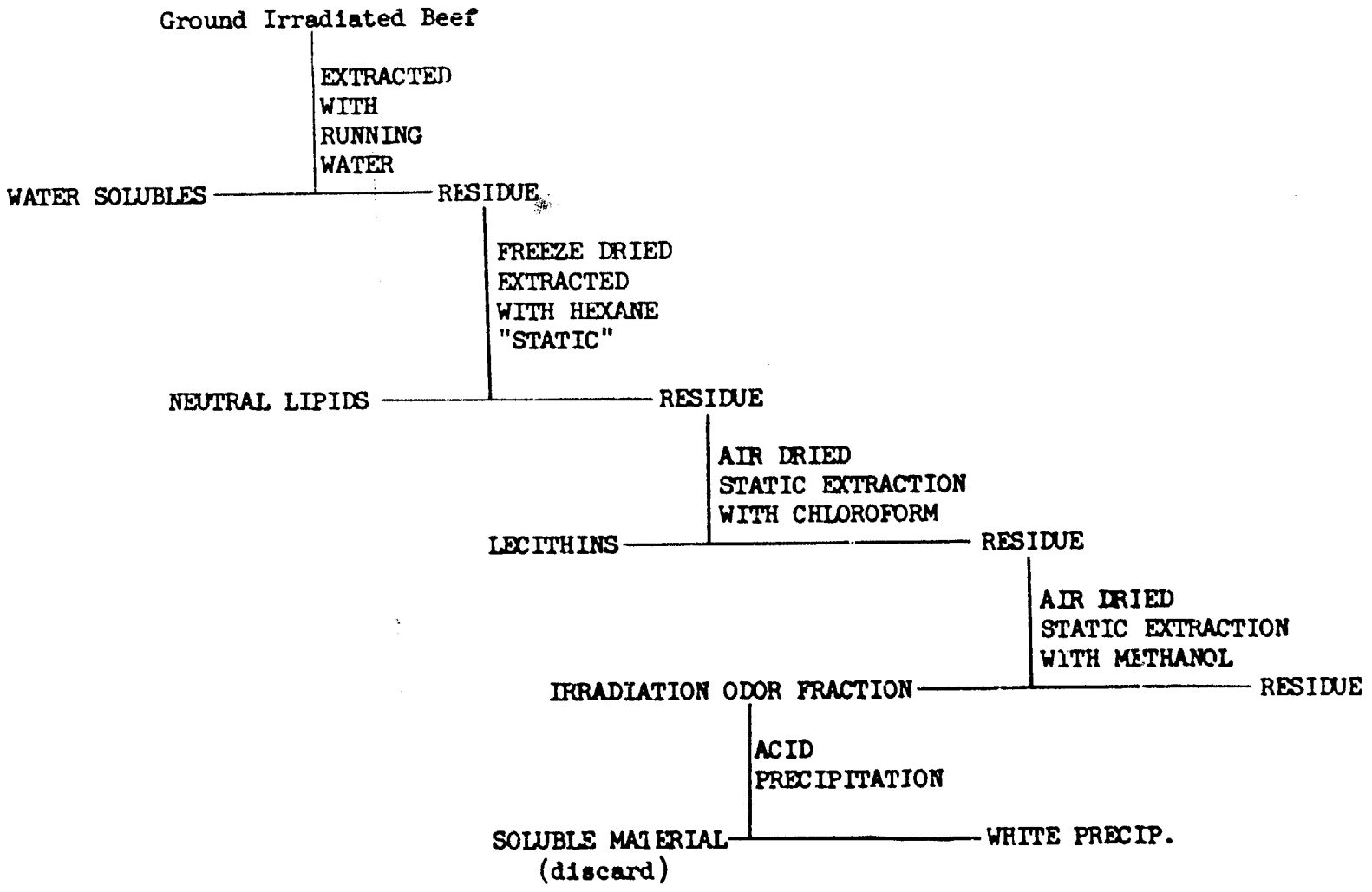
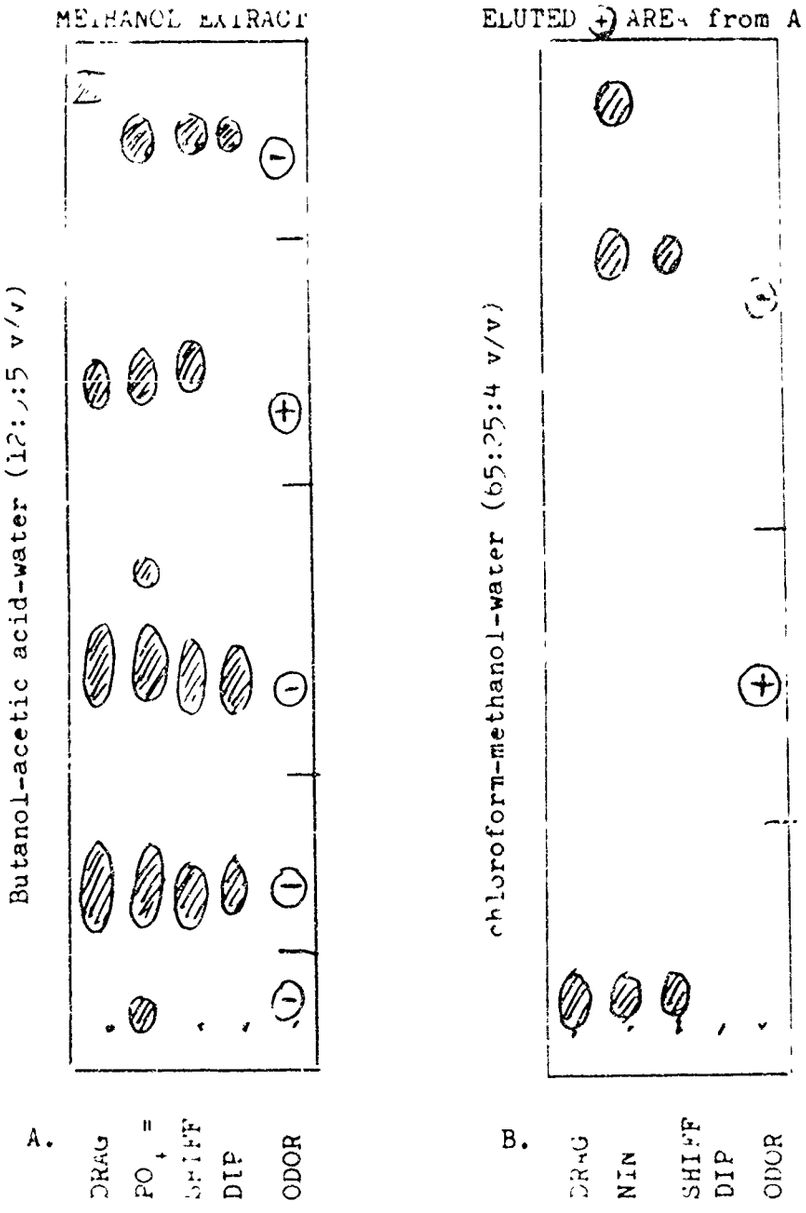


Figure 2

THIN LAYER CHROMATOGRAMS
OF IRRADIATION ODOR MATERIAL



DRAG = DRAGENDORFF'S SOLN.
DIP = DIPHENYL AMINE
NIN = NINHYDRIN

from the base line because no other spots appeared when the plates were treated with the usual reagents. This was a good indication of the degree of purification of the methanol extract by acid precipitation.

A number of solvent systems were tried. Diisobutyl ketone-acetic acid-water 40:20:3 v/v moved the single spot about 1/4 in. from the base line. In order to remove the solvent odors (primarily butyric acid present in the diisobutyl ketone) so that the irradiation odor could be detected, it was necessary to dry the plate for 24 hours and then wash it with distilled water. The washing was accomplished by "re-chromatographing" the plate using distilled water as solvent. No further movement of the previously observed spot occurred. When the silica gel was removed from the spot area of the untreated portion of the plate, the irradiation odor was present. The material gave positive tests for choline, phosphate and aldehyde. Commercial sphingomyelin gave similar results on TLC but did not contain the odor.

Gas Chromatography:

Attempts were made to identify the irradiation odor by use of gas chromatography. Samples of the gaseous odor taken from the head space of a flask containing the acid-precipitated material were injected into the apparatus. A 4 ft. column, 1/4 in. diameter, containing LAC 728 as the liquid phase on Chromosorb W, 100-120 mesh, inert carrier was used. Runs were made at ambient temperature and at 150° C. Both hydrogen flame and ionization detectors were used. In all attempts no peaks were recorded even though the odor could be detected at the exit ports. Attempts were also made to concentrate the odor as a liquid or solid for injection into the gas chromatograph. Efforts at steam or vacuum distillation either resulted in a loss of the odor, or production of new odors not necessarily connected with the irradiation odor. In one experiment, a U-tube was fitted with two 25 ml flasks to form a closed system. Into one of the flasks was placed material which emitted a strong irradiation odor. The other empty flask was submerged in a dry ice-acetone mixture. After 16 hours (overnight) the submerged flask was removed from the freezing bath and the entire apparatus allowed to come to room temperature. The flask which contained the substance with the irradiation odor now had only a very faint lecithin type odor. The other flask which was submerged in the dry ice-acetone mixture contained no detectable odor. We have no explanation to offer for this observation. The experiment was repeated several times with the same results.

Infra red Spectroscopy:

Infra-red analysis of the irradiation odor was also attempted. A sodium chloride gas cell with a 10 cm. path was filled with the irradiation odor by placing some of the acid precipitate at the bottom of the cell out of the light path and allowing the odor to generate. When a spectrum was taken, no peaks were recorded in the range from 2.5 to

15 microns, yet energy transmission was reduced some 20%. The odor in the cell was quite strong and actually could not be completely removed from the cell even after repeated washings with methanol.

To test the sensitivity of the cell, 0.5 ml of the vapor from a bottle of ethanol was injected into the cell, and a spectrum taken. Strong signal responses were obtained in the appropriate regions. The odor of ethanol was barely detectable.

Infra-red analysis was also used in attempts to determine the nature of the substance contained in the acid-methanol precipitate. A thin film of the precipitate was deposited on an IRtran-2 disc by slow evaporation of an alcoholic solution of the precipitate. The spectrum obtained had three peaks at 2830, 2925 and 2980 cm^{-1} which were interpreted as C-H stretching frequencies. Another major peak occurred at 1743 cm^{-1} which could be due to an ester linkage. Minor peaks occurred at 1470, 1430, 1360, 1180, and 1175 cm^{-1}. On searching the literature, this spectrum strongly resembled those for sphingomyelin. A spectrum taken of commercial sphingomyelin was quite similar to the one obtained from the acid precipitate.

Chemical Tests.

The irradiation odor can be trapped or removed from a container in which it is present, by sodium hydroxide solution. This was accomplished by placing a 5 ml. beaker containing 1 ml. 0.1N sodium hydroxide over a solution of the water washed acid-methanol precipitate in a closed beaker. During the time the beaker with the sodium hydroxide remained over the solution, the irradiation odor could not be detected. If the beaker was removed, the odor was again detectable. The 0.1N NaOH solution, after an overnight collection of vapor, was removed, placed in a 15 ml. separatory funnel, acidified with 1N hydrochloric acid, and then extracted with 5 ml. diethyl ether. The ether solution was evaporated to about 3 to 4 drops, transferred to a micro test tube (1 ml.), evaporated to dryness and tested for elementary nitrogen according to the procedure in Feigl (3). The results were always positive. A duplicate control treated under the same conditions in every respect except that no white precipitate was present, gave negative results. As a matter of routine the solutions were also checked for sulfur and were always negative. The question that could not be resolved was whether the nitrogen was an integral part of the irradiation odor, or whether it was coincidental. Attempts to regenerate the odor from the sodium hydroxide solution by acidification were not successful.

The results obtained with TLC and infra-red indicated that sphingomyelin could be the precursor material which produces the odor during irradiation. Some of the earlier results (1) also indicated that the odor was caused by oxidation which was catalyzed by irradiation. Based on these observations, attempts were made to duplicate the irradiation odor by chemical means.

Sphingomyelin (commercial source) was oxidized with 30% hydrogen

peroxide for 30 minutes at room temperature. Crude catalase in water solution was added to stop the reaction. The irradiation odor was easily detectable even though other odors were formed.

In order to determine if the sphingosine moiety of sphingomyelin was the source of the odor, sphingosine sulfate (commercial source) was treated in the same manner as above. The irradiation odor could be detected but it was difficult to determine whether any appreciable amount was formed due to other odors which were present in the preparation prior to oxidation. Unfortunately the purity of the commercial preparation of sphingosine was quite doubtful, but since no better material was available, there was no alternative but to use the impure material.

Fresh non-irradiated muscle tissue was also carried through the usual extraction procedure to obtain the counterpart of the acid-methanol precipitate from irradiated meat. This material was oxidized with hydrogen peroxide and the reaction stopped with catalase. Again, the irradiation odor was formed.

Other methods of oxidation were tried on these materials. Dilute solutions of acid, neutral, and alkaline permanganate produced quite an array of odors, none of which resembled the irradiation odor. Ozonolysis was tried. Air was passed through a spark gap ozone generator and bubbled through water suspensions of sphingosine, sphingomyelin and acid-methanol precipitate from non-irradiated meat. The results obtained by ozonolysis were quite similar to that of hydrogen peroxide oxidation for all the materials used.

The results with hydrogen peroxide and ozonolysis suggested that the the unsaturation points in fatty acid may be the point of oxidative attack resulting in the odor production. To test this specifically, lipoxidase (Worthington Biochemical Corp.) was tried in the following manner: 10 mg. sphingomyelin (commercial source) was suspended in 50 ml. 0.2 M borate buffer, pH 9.0 with a magnetic stirrer. One ml. of the enzyme solution (~20 ug/ml.) was added and the odors noted from time to time. A strong "lake breeze" type odor (similar to old oxidized linolenic acid) developed immediately, followed by the irradiation odor (~15 minutes later). The "lake breeze" type odor was so pronounced that it was difficult to estimate the intensity of the irradiation odor. The possibility that the "lake breeze" odor may be a component of the irradiation odor was reported earlier (4). The association of the two odors has been noted upon numerous occasions, and may be due to the presence of a common structure. The "lake breeze" odor has been identified with certain unsaturated aldehydes which can be formed from linolenic acid. Perhaps similar compounds can form from the fatty acids in sphingomyelin or the sphingosine structure. Thus one could account for the presence of the "lake breeze" odor either as being a separate entity which is formed at the same time as the irradiation odor, or formed somewhere during the sequence of oxidation which gives the irradiation odor; or which is an integral part of the irradiation odor molecule.

Sphingomyelin and sphingosine sulfate (both commercial preparations) in the dry state, and suspended in water, were given a 5 megarad dose

(at Natick facility). After irradiation, water was added to the dry samples, and all samples checked for odor. A variety of odors were present but none resembled the irradiation odor. Also, the odors were quite different from those obtained with peroxide and lipoxidase.

Discussion:

Based on TLC and infra-red results, and the partially successful results with peroxide oxidation, the indication that sphingomyelin or some substance quite similar to it may be the precursor material for the compound(s) that are responsible for the irradiation odor when beef muscle tissue is subjected to a sterilizing radiation dosage. Some of the results obtained with other experiments does not bear this out, but the variability of conditions could account for them. In the experiments where sphingomyelin and sphingosine were irradiated, and the irradiation odor was not produced, could be attributed to the difference in conditions. To produce the odor from these substances in intact muscle tissue requires different conditions than those used in irradiation of the "pure" substance. A lower dose may be required under these conditions.

Another factor that requires consideration is that commercial sources of sphingomyelin are not necessarily the same substances as those found in intact muscle tissue. If the fatty acid moiety of sphingomyelin is the substance that gives off the irradiation odor the fatty acid may be a very specific one. In most of the commercial preparations of sphingomyelin, hydrolysis with 0.2N sodium hydroxide by refluxing for several hours, is used to remove hydrolecithins. This seemingly mild treatment however, is sufficient to cause, at least, a rearrangement of double bonds in unsaturated fatty acids, so that the commercial material may not give as intense an odor as that present in muscle tissue.

The necessity for obtaining unaltered sphingomyelin for use in these experiments was obvious. Although sufficient time to do this was not available before termination of this work, one procedure (5) for isolation of the material from lung tissue was tried. During several stages of the procedure a very intense irradiation odor was noted, even though unirradiated lung tissue was used. However, due to ambiguity in the instructions, the bulk of the sphingomyelin was lost.

Conclusion:

On the basis of the various observations made during the course of this work, there is a definite indication that sphingomyelin or some compound closely related to it, is the precursor material for the specific irradiation odor that was dealt with here. The nature of the odor itself was not determined.

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Irradiation odor	9		9		9		9	
Beef	9		9		9		9	
Muscle tissues	9		9		9		9	
Precursor materials	9							
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